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Process for the ultrafiltration of biological matrices comprising peptides or proteins

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(56) Related Art  
EP 311283  
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**Abstract**

**Process for the ultrafiltration of biological matrices comprising peptides or proteins**

The present invention relates to a process for the prepurification, by means of ultrafiltration, of complex biological matrices, in particular of fermentation broths, which comprise peptides or proteins.

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**ORIGINAL  
COMPLETE SPECIFICATION  
STANDARD PATENT**

Application Number:

Lodged:

Invention Title:

PROCESS FOR THE ULTRAFILTRATION OF BIOLOGICAL MATRICES  
COMPRISING PEPTIDES OR PROTEINS

The following statement is a full description of this invention, including the  
best method of performing it known to us :-

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**Description**

Process for the ultrafiltration of biological matrices including peptides or proteins.

5 The present invention relates to a process for prepurification, by means of ultrafiltration, of complex biological matrices, in particular fermentation broths, which include peptides or proteins.

To isolate and purify proteins or peptides from fermentation broths, for example  
10 by means of chromatographic methods, a prepurification must first be carried out, this including desalination in many cases.

One example of this is the isolation of the thrombin inhibitor hirudin, a single-chain polypeptide with 65 amino acids, from the culture supernatant of a strain of the  
15 yeast *Saccharomyces cerevisiae* modified by genetic engineering.

The polypeptide hirudin, originally isolated from the leech *Hirudo medicinalis*, is a highly specific thrombin inhibitor with a broad therapeutic potential (F. Markward, Biomed, Biochim. Acta 44 (1985) 1007-1013). However, the amounts required  
20 can be prepared only by a genetic engineering route via transformed microorganisms. It has been found in this context that the yeast *Saccharomyces cerevisiae* is a suitable host organism for producing correctly folded and fully active hirudin (EP A1 168 342, EP A1 200 655). Secretion of the protein results in concentrations of up to a few hundred milligrams of hirudin per liter of culture  
25 filtrate. However, a high yield of the protein can be achieved only if complex nutrient media with the addition of yeast extract, corn steep, peptone or meat paste are employed during fermentation of the yeast, so that, for purification of the protein, there is the problem of isolating hirudin from a high dilution in a mixture of protein-like concomitant substances.

30



- In addition to conventional methods, such as, for example, extraction or precipitation, hydrophobic adsorptions/desorptions, for example on non-polar polymer materials (also HIC), are also used for desalination of the culture broths thus obtained and prepurification in preparation for
- 5 chromatography stages (Atkinson, F. Mavituna; Biochemical Engineering and Biotechnology Handbook, Chapter 16 "Downstream Processing" and Chapter 17 "Product Recovery Processes and Unit Operations", Second Edition, Stockton Press 1991, New York, U.S.A; Brocklebank, M. Kalyanpur: "Primary Separation", Chapter 4 in G. Schmidt-Kastner et al.,
- 10 editors: "Recovery of Bioproduct", European Federation of Biotechnology, Study Report of Working Party on Downstream Processing, 1993; Müller and W. Brümmer: "Die Chromatographie, eine zentrale Methode in der biotechnischen Aufarbeitung" [Chromatography, a central method in biotechnical workup], Chem. Ing.-Tech. 62 (1990) No. 5, pages 380-390).
- 15 These processes usually have the feature that in some cases large amounts of solvents or salts are employed, compared with the amount of product to be prepared. This leads to additional costs or to increased technical complexity for recovery and/or disposal of the solvents or salts. Furthermore, the spent adsorption resins have to be gotten rid of as waste.
- 20 In contrast, the object of the present invention is to provide, for prepurification, in particular for desalination and concentration, of peptide- or protein-containing fermentation broths, a membrane ultrafiltration process which is suitable for desalinating and concentrating the culture
- 25 broth to a degree required for the subsequent purification steps, with high retention of the peptides or proteins.
- 30 Ultrafiltration processes for the prepurification of fermentation broths in preparation for chromatography stages have not been employed to date on a large industrial scale on these early process stages - apart from for the purpose of removal of cells (T.J. O'Sullivan et al. Chem. Eng. Prog. 80 (1), 68-75 (1984); A. Erikson, Desalination, 53 (1985), 259-263). One reason for this is that the membranes are often blocked by the by-products and concomitant substances, which may be diverse in nature, and therefore

lead to permeate flow rates which - for production purposes - are unsuitably low; additional problems therefore also result in the cleaning or regeneration of the membranes (Winzeler: "Membran-Filtration mit hoher Trennleistung und minimalem Energiebedarf" [Membrane filtration with a high separation efficiency and minimal energy requirement], *Chimia* 44 (1990) 288 - 291). This particularly applies to preparation processes for proteins of low molecular weight ( $M < 50,000$  Dalton) and peptides, which supposedly require ultrafiltration membranes with very low separation limits (molecular weight cut-off).

10

Only at later process stages where prepurification has already taken place, for example between successive chromatography stages, are ultrafiltrations, for example for desalination and concentration, already prior art in production.

15

Ultrafiltration processes by means of membranes are furthermore used for separation of proteins of different size, for removal of pyrogens or for isolation of biocatalysts (T.J. O'Sullivan et al., *Chem. Eng. Prog.* 80(1), 68-75 (1984); E. Flaschel et al., *Adv. in Biochem. Engineering/Biotechnologie* Volume 26, "Downstream Processing", pages 73-142, N.Y. 1983; Editor: D.J. Bell).

20

The processes mentioned are all based on the principle of employing, for separating off a peptide or a protein, a membrane of separation limit (molecular weight cut-off) in or below the molecular weight range of the peptide or protein to be retained.

25

It has now been found that it is entirely possible to desalinate and concentrate a protein or peptides having a low molecular weight with very high retention of the product on membranes which have a nominal separation limit (molecular weight cut-off) of up to 30,000 Dalton.

30

The present invention accordingly relates to a process for prepurification of a cell-free culture broth including peptides or proteins by means of



ultrafiltration on a membrane, wherein the stated separation limit of the membrane is two to five times, preferably three to four times, the molecular weight of the peptide or protein to be retained by the membrane.

- 5 The present invention is used in particular for prepurification of cell-free culture broths which include a recombinant hirudin, in particular such a hirudin which is expressed in *Saccharomyces cerevisiae*.

- 10 Hirudin is to be understood as meaning peptide-like thrombin inhibitors having a specific activity of at least 1000 AT-U/mg, which are derived from the known isohirudins of the species *Hirudo medicinalis* and have the essential structural features of these, in particular the characteristic linkage of the three disulfide bridges (J. Dodt et al., *Biol. Chem. Hoppe-Seyler* 366 (1985) 379-385); (cf. for example EP A1 158 564, EP A1 168 342, DE 34 45 517, EP A2 193 175, EP A1 200 655, EP A1 158 986, EP A1 209 061, DE 33 42 199, EP A1 171 024).

In particular, this is understood as meaning a hirudin such as is described in EP A1 171 024, EP A1 158 986 and EP A1 209 061.

- 20 The process according to the invention is particularly preferably employed for prepurification of a cell-free culture broth which includes a hirudin derivative having the amino acid sequence disclosed in EP 0 324 712 ((Leu<sup>1</sup>, Thr<sup>2</sup>)-63-desulfohirudin).

- 25 The separation limit of the membrane for prepurification of hirudin-containing culture broths is 20 to 30 kD, preferably 20 kD.

- 30 The specific permeate flow rates in the process according to the present invention are preferably 10 to 35 l/m<sup>2</sup>/h over the entire course of the filtration.

If corn steep is used in the fermentation medium, the temperature of the culture broth is preferably 5 to 15°C.



In the process according to the invention, the permeate is preferably recycled to the crude solution until steady-state conditions have been established, manifested by the fact that the product concentration in the permeate no longer rises, i.e. is constant.

5

#### Example

At the end of the fermentation of a strain of *Saccharomyces cerevisiae*, modified by genetic engineering, in a complex medium comprising corn steep and yeast extract for preparation of hirudin,  $0.225 \pm 0.025\%$  of benzalkonium chloride, for example  $0.45 \pm 0.05\%$  of Dodigen® 226 (a 50% strength solution of a mixture of alkyldimethyl-benzyl-ammonium chlorides in water), is added to the culture to inactivate the cells and the mixture is incubated for 30 minutes.

15

The broth is then passed via a separator or decanter to remove the cells, and is then clarified by a 2-stage layer filtration in a filter press comprising a fine and a sterilizing stage. The filtrate is cooled to temperatures of  $T \leq 15^\circ\text{C}$  to avoid renewed temperature-related precipitations, which are reversible.

20

The filtrate thus prepared has a conductivity of  $\kappa = 6 \pm 0.5 \text{ mS/cm}$ . The ultrafiltration unit, fitted with membranes of cellulose acetate having an exclusion limit of 20,000 Dalton (for example Nadir® UF-CA-20 in the form of 3.8 inch spiral coil modules), is first set in operation under the following conditions for 60 minutes, with recycling of the permeate:

25

average transmembrane pressure	$4 \pm 1 \text{ bar}$
volume flow per pressure tube	$4 - 5 \text{ m}^3/\text{h}$

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The filtrate is then concentrated to a degree of 6:1 under the same conditions, with removal of permeate, and is subsequently subjected to diafiltration, i.e. desalinated, by addition of demineralized and filtered water (purified water), while keeping the volume constant, until the conductivity  $\kappa$

is  $\leq 2.0$  mS/cm. After the diafiltration, the product can be concentrated again, in order to arrive at a concentration of 8:1 in total; a conductivity of  $x < 2.2$  mS/cm should be maintained here.

5 Process data and results:

	Initial volume	:	4600 l
	Initial concentration of hirudin	:	100%
	Final volume	:	710 l
10	Final concentration of hirudin	:	607.2%
	Initial conductivity	:	5.83 mS/cm
	Final conductivity	:	1.97mS/cm
	Yield of hirudin	:	93.7%
	Recovery	:	96.9%
15	Product loss in the permeate	:	3.3%



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for the prepurification of a cell-free culture broth including peptides or proteins by means of ultrafiltration on a membrane, wherein the stated separation limit of the membrane is two to five times the molecular weight of the peptide or protein to be retained by the membrane.
2. The process as claimed in claim 1, wherein the stated separation limit of the membrane is three to four times the molecular weight of the peptide or protein to be retained by the membrane.
3. The process as claimed in claim 1 or 2, wherein the peptide is a hirudin.
4. The process as claimed in claim 3, wherein the separation limit of the membrane is 20 to 30 kD.
5. The process as claimed in any one of claims 1 to 4, wherein the specific permeate flow rates over the entire course of the filtration are 10 to 35 l/m<sup>2</sup>/h.
6. The process as claimed in any one of claims 1 to 5, wherein, if corn steep is used, the culture broth has a temperature of 5 to 15°C.
7. The process as claimed in any one of claims 1 to 6, wherein the permeate is recycled until the product concentration in the permeate has assumed a constant value.

DATED this 23rd day of May 2000

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**PRIOR ART**